Title: The tape measure protein of the *Staphylococcus aureus* bacteriophage vB_SauS-phiIPLA35 has an active muramidase domain.

Running Title: Tape measure protein with muramidase activity.

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Abstract

Tailed dsDNA bacteriophages frequently harbour structural proteins displaying peptidoglycan hydrolytic activities. The tape measure protein from *Staphylococcus aureus* bacteriophage vB_SauS-phiIPLA35 has a lysozyme-like and a peptidase_M23 domain. This report shows that the lysozyme-like domain (TG1) has muramidase activity and exhibits *in vitro* lytic activity against live *S. aureus* cells, an activity that could eventually find use in the treatment of infections.

The peptidoglycan layer is a barrier that bacteriophages must overcome to infect its bacterial host. In this regard, virions are provided with structural proteins containing catalytic domains that play roles in attachment to host cells through sugar binding or degradation of the polysaccharides on the cell surface (6). Murein hydrolases appear to be widespread in virions of bacteriophages infecting Gram-positive and Gram-negative bacteria, where the enzyme is either part of a large protein or found associated with other structural components of the virion (10). These peptidoglycan hydrolases have been related to modification in the peptidoglycan structure necessary for phage infection. Sequence analysis revealed that peptidoglycan degrading domains are frequently located in Tape Measure Proteins (TMPs) (6), although in most of the cases its activity has not been analysed. The TMPs of several mycobacteriophages contain domains that mediate peptidoglycan degradation and play an essential role when these phages infect stationary phase cells (12). In a similar way, phage T5 straight tail fiber is a multifunctional protein not only acting as a tape measure protein but carries also fusogenic and muralytic activities (3). In other phages, virion-associated proteins with peptidoglycan hydrolytic activity might play a similar role in the phage infection process. Thus, the bacteriophage T7 gp16 with transglycosylase activity is not essential for phage growth, but was shown to be beneficial during infection of *E. coli* cells grown to high cell density or low temperatures, where the murein is more highly cross-linked (9). Furthermore, the amino-terminal end of the bacteriophage PRD1 structural protein P7 carries a conserved transglycosylase domain, which is located at the particle vertices and is involved in the early steps of the PRD1 life cycle (16). Additionally, the tail baseplate of bacteriophage T4 contains an essential protein, gp5, which possesses lysozyme activity and functions to locally dissolve the periplasmic cell wall (1).

S. aureus bacteriophage vB_SauS-phiIPLA35 (in short, phiIPLA35) is a member of the Siphoviridae family, previously isolated from dairy samples (7). The complete genome sequence was determined (GenBank EU861005) and zymogram analysis of virions revealed the presence of a phiIPLA35 virion-associated muralytic enzyme (8). In this study, a muramidase domain has been described as part of the TMP, a structural component of phage phiIPLA35. The lytic activity of this protein against *S. aureus* cells has been determined and its cleavage sites in the peptidoglycan identified.

The tape measure protein (TMP) of phage phiIPLA35 has two putative catalytic domains. Computer-based similarity searches using BLASTp revealed that protein TMP (gp50, 2066 aa, YP_002332413.1), showed 98-99% similarity with TMPs from the staphylococcal phages 42E, phi47, phi12, phiSLT, tp310-2, and phi3A. Conserved-domain analyses of TMP using InterProScan identified a structural conserved domain, tape_meas_TP901 (from aa 323-666), related with the tail length in TP901-like phages (11). In addition, two typical catalytic domains were observed: a peptidase_M23 domain (from aa 1706-1796) and a lysozyme-like domain (from aa 1829-1920). Members of peptidase family M23 are zinc metallopeptidases with Gly-Gly endopeptidase activity and many of them such as lysostaphin have specific hydrolytic activity on peptidoglycan (17). Lysozyme activities catalyze the cleavage of the beta-1,4-glycosidic bond between N-acetylmuramic acid (MurNAc) and N-acetyl-D-glucosamine (GlcNAc). Lysozyme catalytic domains are often found in cell wall hydrolases from *S. aureus* phages (14). However, only phages 42E, phi47, phi12, phiSLT, tp310-2, and phi3A revealed a TMP with a lysozyme-like domain homolog to phiIPLA35 TMP.

The lysozyme-like domain (TG1) of TMP has peptidoglycan muramidase activity. To confirm the predicted peptidoglycan hydrolytic activity of TG1, a truncated

78 protein (from aa 1829 to 2066) containing the lysozyme-like domain was cloned and
79 overexpressed. phiIPLA35 *tg1* fragment (717 bp) was codon-optimized based on the *E.*
80 *coli* codon usage and commercially synthesized (Biomedal, Sevilla, Spain). Then *tg1-*
81 *opt* was subcloned between the *Nde*I and *Xho*I sites in the multi cloning site of the
82 inducible expression vector pET21a (EMD Biosciences, San Diego, CA), which
83 introduces a C-terminal 6xHis-tag. *E. coli* BL21(DE3)/pLysS was used in protein
84 expression assays (19). Purification of TG1 (30.1 kDa) was performed by Ni-NTA
85 chromatography (15). Peptidoglycan cleavage site determination was carried out by
86 reverse-phase HPLC and Mass Spectrometry (MS) (2). 2 mg of *S. aureus* SA113
87 peptidoglycan was isolated by CeCoLabs UG, Tübingen, incubated overnight with 27
88 µg of TG1 in a final volume of 250 µl at 37ºC with shaking in MES-NaOH 50 mM, pH
89 5, buffer. Samples were boiled 3 min to stop the reaction, centrifuged at 14,000 × *g* for
90 5 min and the soluble fraction was separated by reverse-phase HPLC using a Nucleosil
91 100 column (C18; 125 × 4.6 mm; 5 µm; Maisch GmbH, Ammerbuch, Germany) and a
92 water/0.1% TFA: 80% acetonitrile/0.1% TFA gradient for 150 min at flow rate of 0.5
93 ml/min. The molecular masses of the muropeptides were determined by LC-MS. The
94 primary product of TG1 digestion was the disaccharide pentapeptide GlcNAc-(β1–4)-
95 MurNAc-(L-Ala-D-iGln-L-Lys-(Gly)-D-Ala-COOH) (M_r 951.7) which in MS yields a
96 peak with *m/z* 952.6 (protonated) and *m/z* 950.5 (unprotonated) (Fig. 1). This main
97 digestion product is also obtained with the mutanolysin digestion of the same
98 peptidoglycan (data not shown). Therefore, TG1 is most likely a muramidase that can
99 digest MurNAc-GlcNAc linkages.

100 There are no previous reports about the involvement of virion-associated
101 peptidoglycan hydrolases in *S. aureus* phage infection. However, the presence in phages
102 phiIPLA88 and phiMR11 of proteins with muralytic activities might indicate its

involvement in local cell-wall degradation, allowing the subsequent introduction of DNA into the host cytoplasm (13, 14). In phiIPLA35, the presence of an active muramidase domain in the TMP could also indicate its contribution in the infection process, taking into account that no other virion-associated proteins with peptidoglycan hydrolase activity were identified in this phage (8).

The TG1 domain displays lytic activity against *S. aureus* cells. The peptidoglycan hydrolytic ability of TG1 was assayed by zymogram analysis using 10 ml 15% (w/v) SDS-PAGE with or without *S. aureus* Sa9 cells from a 300 ml culture (A_{600nm} 0.5) embedded in the gel . A clear band consistent with the predicted molecular mass (30.1 kDa) was observed after analysis of 5 μg of TG1 protein in the SDS-PAGE containing the staphylococcal cells. This clear band was the result of the lysis of the *S. aureus* cells embedded in the gel (Figure 2A, zymogram). Moreover, in order to assess the lytic activity of TG1 against live *S. aureus* Sa9 cells, turbidity reduction assays were performed with *S. aureus* cells grown to logarithmic phase (OD_{600nm} = 0.4–0.6), harvested and resuspended in buffer A (MES-NaOH 50 mM, pH 5) to OD_{600} 1.0. As shown in Figure 2B, a 32.7% decrease in OD_{600nm} was obtained after 120 min of incubation at 37ºC with 5 μM of TG1 (specific activity 0.00138 expressed as ΔOD_{600nm} $min^{-1}\mu M^{-1}$), which indicated the lytic ability of the TG1 protein. This result was confirmed by a cell viability assay using exponentially growing cells which were recovered by centrifugation, washed and resuspended in buffer A to OD_{600} 0.1. Then, 5 μM TG1 was mixed with *S. aureus* Sa9 live cells to a final concentration of 4×10^6 CFU/ml and incubated for 30 min, 1, 2 and 3 h at 37 ºC. Staphylococcal viable counts were reduced by 75.5%, 77.5%, 98% and 99.7%, respectively, compared with the untreated control cultures (Figure 2C). These results support the functionality of the putative lysozyme-like domain found by the bioinformatic analysis. Nevertheless, their

activity seems to be somewhat weaker than that shown by other staphylococcal virion-associated peptidoglycan hydrolases such phiIPLA88 HydH5 (14) and phiMR11 gp61 (13). This lower specific activity (about 23-fold lower compared to HydH5) could be due to the presence of only one catalytic domain, the lysozyme-like domain, while HydH5 showed two active catalytic domains (14). Nevertheless, the peptidoglycan hydrolytic activity of TMP as part of phiIPLA35 virions could also be enhanced by the peptidase_M23 domain. Therefore, although TG1 lytic activity was moderate against staphylococci, further investigation is worthwhile. We previously reported that the fusion of a staphylococcal SH3b cell wall-binding domain to the HydH5 CHAP domain resulted in a 4.8-fold increase of its lytic activity (15). Furthermore, it is plausible to swap different catalytic domains by protein engineering generating proteins with improved lytic activity (4, 15). In this context, the search of catalytic domains with new biochemical properties is justified. Moreover, with the increasing prevalence of antibiotic-resistant pathogens, the possibility of using phage lytic proteins as novel therapeutic antibacterial agents has a renewed interest. This is not only supported by its ability to kill bacteria but also by the low probability of resistance development. In fact, some phage endolysins have already been successfully assayed in preclinical trials involving animal models of human diseases (5). Additionally, chimeric lysins consisting of an endopeptidase domain of the streptococcal phage lysin λSA2 and SH3b cell binding domains from the staphylococcal phage lysin LysK and lysostaphin showed antimicrobial activity against *S. aureus*-induced mastitis in mouse models (18).

In conclusion, the identification of a muramidase activity associated to the phage phiIPLA35 tape measure protein showing lytic capabilities against live *S. aureus* cells is reported. This novel peptidoglycan lytic domain may be of help for designing potent antimicrobials against this pathogen.

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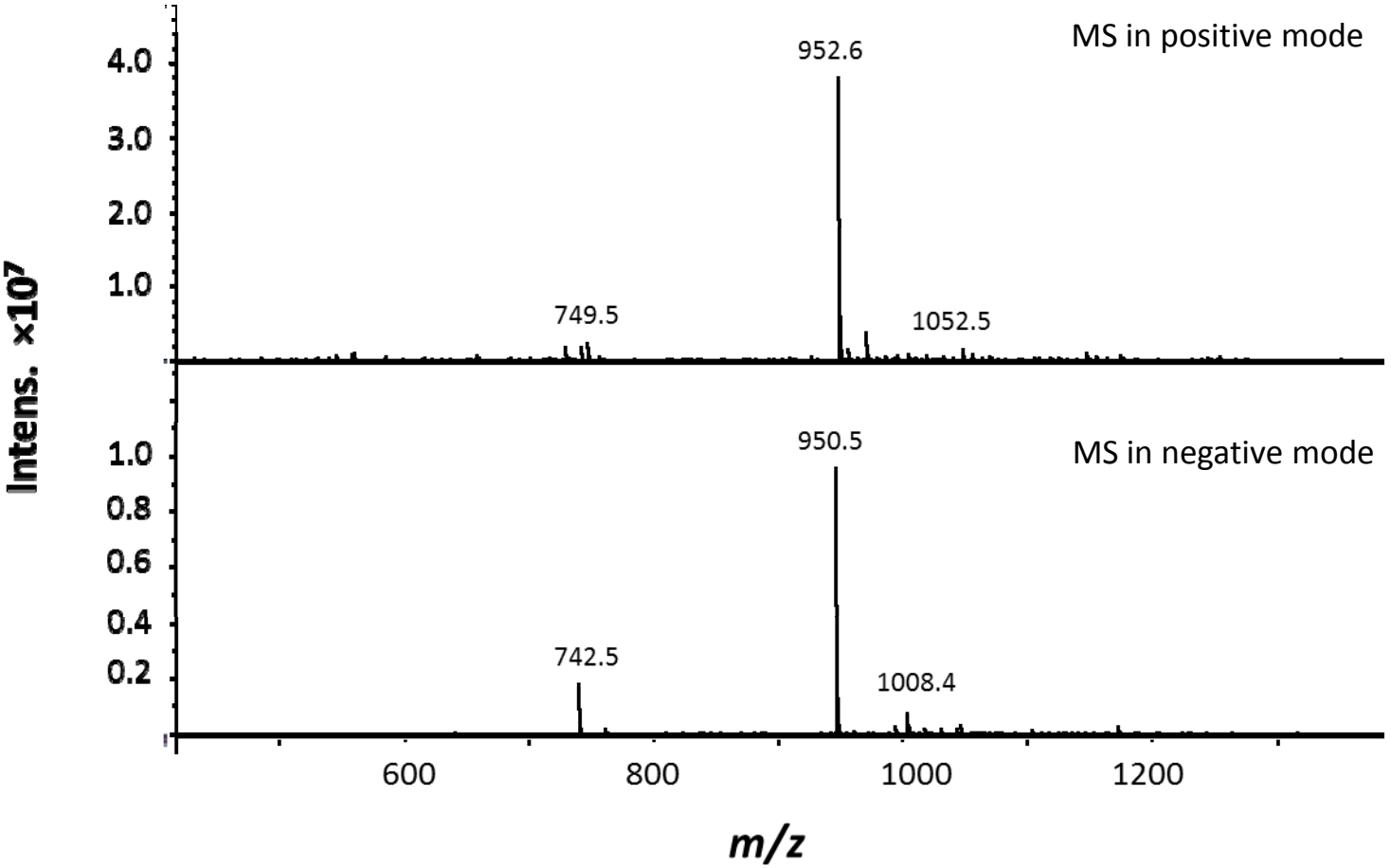
Figures

Figure 1. MS analysis of the overnight digestion of 2 mg of *S. aureus* SA113 peptidoglycan with 27 µg of TG1 in MES-NaOH 50 mM, pH 5 buffer. The primary product of the digestion was the disaccharide pentapeptide GlcNAc-(β1–4)-MurNAc-(L-Ala-D-iGln-L-Lys-(Gly)-D-Ala-COOH) (M_r 951.7)

Figure 2. Lytic activity of TG1 protein against *S. aureus*. A) 5 µg of nickel affinity purified TG1 (30.1 kDa) were resolved on a 15% SDS-PAGE in the absence (SDS-PAGE, lane 1) or in the presence (zymogram, lane 2) of *S. aureus* Sa9 cells. Gels were either stained with Coomassie.blue (SDS-PAGE) or incubated in water for 1 h at room temperature (zymogram). Lane M: Standard molecular mass marker in kDa (Prestained SDS-PAGE Standards, broad range, BioRad Laboratories). B) Representative turbidity reduction assay performed by challenging *S. aureus* Sa9 cells to 5 µM of TG1 for 120 min at 37 ºC. Light grey line indicates the untreated cultures (*S. aureus* Sa9 cells + MES-NaOH 50 mM, pH 5); dark grey line indicates treated cultures (*S. aureus* Sa9 cells + 5 µM of TG1). C) Viability test performed by challenging *S. aureus* Sa9 cells to 5 µM of TG1 for 30 min, 1, 2 and 3 h at 37 ºC (light grey bars). Dark grey bars indicate untreated cultures (*S. aureus* Sa9 + MES-NaOH 50 mM, pH 5). Error bars are the means ± standard deviations of two independent assays.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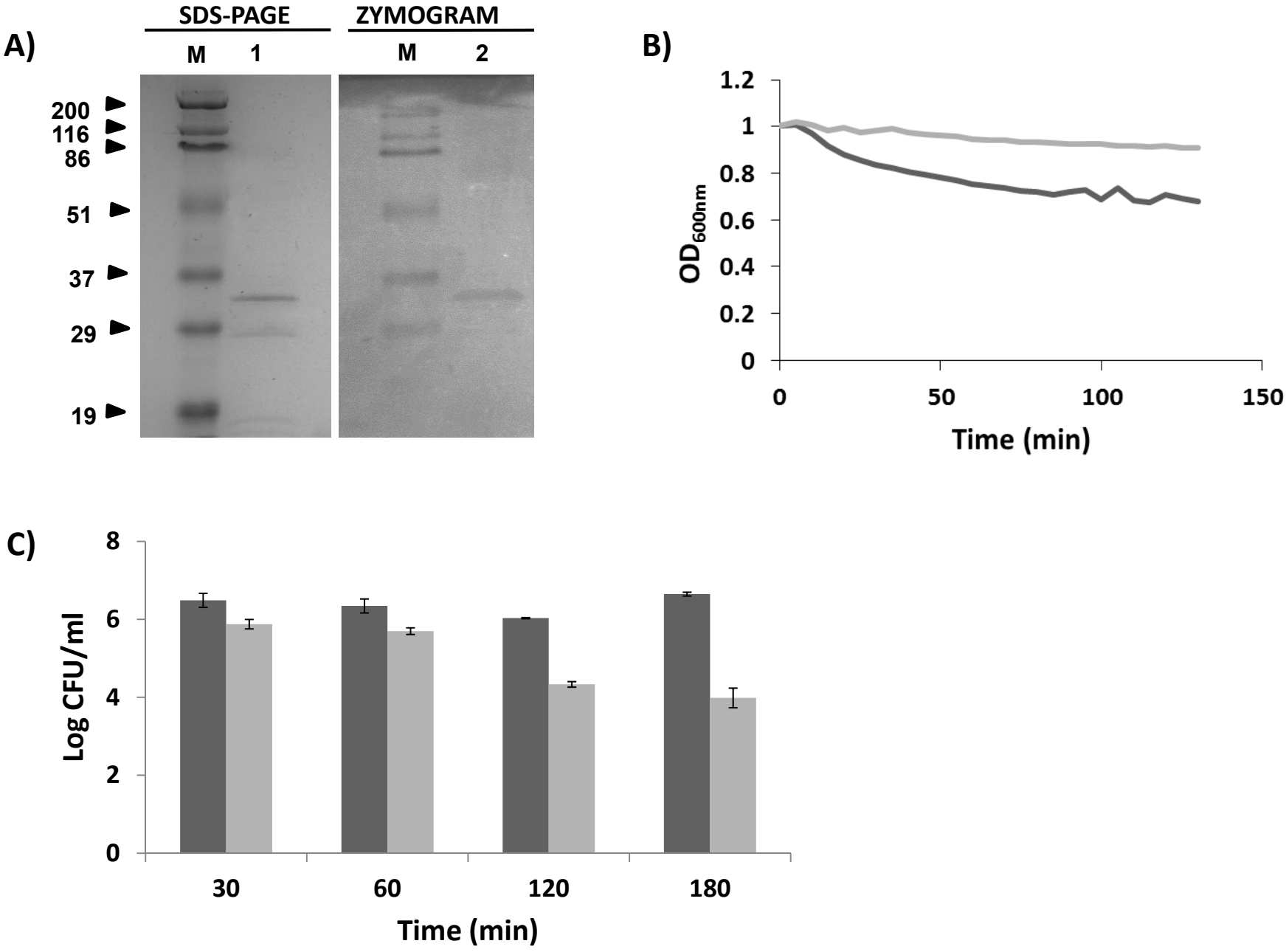
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